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REVIEW

THE ROLE OF GENETIC POLYMORPHISMS IN ALCOHOLIC LIVER DISEASE

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Abstract — Chronic alcohol consumption is a major cause of liver cirrhosis which, however, develops in only a minority of heavy drinkers. Evidence from twin studies indicates that genetic factors account for at least 50% of individual susceptibility. The contribution of genetic factors to the development of diseases may be investigated either by means of animal experiments, through linkage studies in families of affected patients, or population based case–control studies. With regard to the latter, single nucleotide polymorphisms of genes involved in the degradation of alcohol, antioxidant defense, necroinflammation, and formation and degradation of extracellular matrix are attractive candidates for studying genotype–phenotype associations. However, many associations in early studies were found to be spurious and could not be confirmed in stringently designed investigations. Therefore, future genotype–phenotype studies in alcoholic liver disease should meet certain requirements in order to avoid pure chance observations due to a lack of power, false functional interpretation, and insufficient statistical evaluation.

INTRODUCTION

Alcoholic liver disease (ALD) accounts for >50% of all chronic liver diseases in industrialized countries and is responsible for >50 000 annual deaths due to cirrhosis and associated complications (Corrao *et al*., 1997; Kim *et al*., 2002; John and Hanke 2002).

ALD comprises various degrees of liver injury due to direct and indirect effects of continuous exposure towards toxic amounts of alcohol, including alcoholic fatty liver, alcoholic steatohepatitis, as well as alcohol-induced hepatic fibrosis and cirrhosis, either with or without inflammation (Ishak *et al*., 1991). Moreover, chronic alcohol consumption is an established risk factor for the development of hepatocellular carcinoma in patients with liver cirrhosis (Stickel *et al*., 2002; Morgan *et al*., 2004). While nearly all heavy drinkers reveal fatty liver, 10–35% of alcoholics are diagnosed with alcoholic hepatitis, and 10–20% develop cirrhosis (Teli *et al*., 1995). Hence, only a minority of heavy drinkers develop severe liver disease. The cause for this variable susceptibility towards alcohol is yet unclear.

As with other chronic liver diseases, an alcoholic individual's risk to develop ALD is governed by a complex interplay of numerous genes with several known or unknown environmental factors. For example, it is well established that the amount of alcohol consumed is highly correlated with evolution of cirrhosis (Bellentani *et al*., 1997). In addition, alcoholics with chronic hepatitis C virus infection or obesity display more severe liver damage (Wiley *et al*., 1998; Raynard *et al*., 2002). However, these factors are not sufficient to explain the wide diversity of hepatic damage suggesting a role of certain host factors. Accordingly, female gender and the presence of the hemochromatosis gene mutation were identified to increase the likelihood of alcohol-related hepatic damage. Evidence has accumulated supporting the concept that genetic factors unrelated to gender contribute to the emergence of ALD (Hrubec and Omenn, 1981). For example, twin studies have shown that the concordance for alcoholic cirrhosis is significantly greater in monozygotic than in dizygotic twins, leading to the conclusion that ~50% of the phenotypic variation of alcoholism can be attributed to genetic modifiers (Reed *et al*., 1996). The identification of these factors would improve our understanding of the pathophysiology of ALD and greatly help managing affected patients. Identification of genetic factors indicative of rapid disease progression would augment preventive strategies and the timing of therapeutic interventions including liver transplantation. Identified genetic risk factors may also play a role in the progression to cirrhosis in other chronic liver diseases as it represents a common end point of many chronic liver diseases.

METHODOLOGY AND ANALYSIS OF GENETIC STUDIES

While some diseases, such as cystic fibrosis, alpha-1 antitrypsin deficiency, and phenylketonuria, are related to mutations of single gene loci inherited according to Mendelian rules, ALD and the majority of other liver disease are polygenic and represent 'complex traits'. Considerable efforts are required to determine the role of a single or a group of genes, since their effects on disease manifestation are usually smaller than crucial gene defects in monogenic disorders.

Genetically modified animals may be useful in the analysis of disease-specific gene loci either through using knockout mice deficient for the gene of interest, or conversely, transgenic mice which overexpress genes that are relevant for the manifestation or progression of certain diseases (Hillebrandt *et al*., 2003). Recently, an elegant technique termed 'quantitative trait loci' (QTL) analysis has been introduced which combines molecular biology tools with classical approaches of genetics to search for genetic determinants which so far had not been linked to a disease's pathogenesis (Darvasi, 1998; Korstanje *et al*., 2002). In QTL

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analysis, all crossbred animals in an experiment are both phenotyped with regard to a certain quantitative marker and genotyped through a 'genome-wide scan' for markers with known localization within the genome. Using this technique, Hillebrandt *et al*. (2002) have described a susceptibility locus on chromosome 15 that significantly affects the stage of fibrosis in fibrosis-susceptible BALB/c mice. Excitingly, the same group later confirmed their findings by showing that the corresponding gene in humans which codes for complement factor 5, is in fact a quantitative trait gene that modifies liver fibrosis in patients with chronic hepatitis C (Hillebrandt *et al*., 2005).

The detection of disease-modulating genes in humans is more difficult and challenging. Currently, three different approaches have been applied: family-based linkage analyses, candidate gene association studies, and genome-wide polymorphism studies. It is beyond the scope of this review to describe family-based linkage and genome-wide scanning analyses and to dissect their advantages and disadvantages in detail, and therefore, readers are referred to concise review articles focused on this issue (Tabor *et al*., 2002; Day, 2003). Here, emphasis will be exclusively put on genotype–phenotype associations studies.

In humans, candidate gene case–control studies investigating associations between single nucleotide polymorphisms (SNPs) and certain disease end-points have been the most extensively applied method (Hirschhorn *et al*., 2002). In this kind of study, the frequency of one or several polymorphisms are compared between a group of patients displaying the phenotype of interest and another group of unaffected controls exposed to the same insult, e.g. alcohol or viral hepatitis (Gambaro *et al*., 2000; Day, 2003). An association with the disease is assumed should there be a significantly higher frequency of one allele or genotype in either of the groups. Genotype–phenotype studies most commonly focus on the role of SNPs. In an SNP, a single base is substituted for another one thereby leading to an altered base triplet that potentially could code for a different amino acid when located in the coding sequence. In effect, this could result in an altered function of the generated protein. Also, some SNPs result in altered quantitative transcription of the respective gene if located in the promoter sequence. However, most polymorphisms are situated in non-coding regions of genes and, therefore, have little or no impact. This has to be taken into account, when choosing polymorphisms for association studies. SNPs are the most common type of allelic variation and can be found throughout the human genome at a frequency of 1 every 1000–2000 bp (http://www.ncbi.nlm.nih.gov/SNP). So far nearly three million of them are described. Genotyping of SNPs is possible through electrophoresis-based techniques, such as restriction fragment length polymorphism (RFLP) analysis in which a restriction enzyme cuts a PCR product at the site of the SNP thereby generating DNA fragments of different sizes which are then made visible by agarose gel electrophoresis. Since only a proportion of SNPs can be detected through RFLP, PCR techniques using sequence-specific primer pairs that solely amplify the corresponding wild-type or mutated DNA template have been established. Other experimental tools are more complex and include oligonucleotide ligation assays, single-strand conformational polymorphism analysis, or DNA sequencing. While all these techniques are appropriate for small-scale studies on single or a limited number of SNPs, high-throughput genotyping platforms are required to study larger cohorts and multiple SNPs such as *in silico* mapping (Wang and Rannala, 2005), fluorescent dye-based genotyping (Wilson *et al*., 1990), DNA microarrays (Lau *et al*., 2005), and mass spectrometry technologies (Younossi *et al*., 2005).

The selection of a candidate gene for an association case–control study is usually based on biological plausibility in chosen genes that play a putative role in the pathogenesis of the studied disease (Day, 2003). With regard to ALD, it is important to separate genes that are related to alcoholism from those that affect progression of liver disease. In addition, some genes are known to be mutated in a hereditary type of the disease and, therefore, become an interesting candidate for a case–control study that investigates the sporadic type of the disorder. As mentioned above, genes that were identified through research with knockout or transgenic animals are ideal candidates to be further studied in human cohorts. Novel candidate genes can also be identified through DNA microarrays, and then screened for in larger cohorts of affected individuals. After a gene has been selected to serve as a candidate, it should be clarified whether SNPs with a functional implication reside in the corresponding gene. Only genetic variants that result in altered transcription, RNA stability, or protein function are likely to modulate disease progression (Daly, 2003). So, the functional implications of a SNP or a haplotype (a cluster of gene loci that co-segregate) should be characterized through *in vitro* and *in vivo* evidence prior to their testing in association studies.

An important task in genotype–phenotype association studies in ALD is the selection of appropriate cases and controls matched for potential modifiers of liver damage. Therefore, a suitable recruitment strategy should control for potential confounders of an association such as age and gender, extent of alcohol exposure, co-morbidities and co-medication, and ethnicity. These attempts should be made in order to avoid a confounding effect by population stratification due to a marked variation of the allele frequency of certain genes among subgroups with a different baseline risk for the disease (Cardon and Palmer, 2003). Both cases and controls need to be well characterized, and subjects with a similar alcohol consumption and a near normal histology on liver biopsy represent ideal controls. Obviously, this is a difficult task in many instances, so alternatively, controls should at least have normal liver-specific laboratory results and a normal appearance of some type of liver imaging, e.g. ultrasound (Day, 2003).

Statistical issues have become increasingly complex in genetic studies and, particularly, statistical power is important since it reflects the probability that a statistically significant effect is demonstrated when it really exists. Currently, a power of 0.8 is generally accepted for genetic association studies which translates into a 80% chance of detecting a true association (Bataller *et al*., 2003). In 'underpowered' studies, both a type I and a type II error may occur. Type I errors refer to false-positive associations frequently seen in studies with a low sample size, whereas type II errors represent false-negative findings that may result from insufficient patient characterization or population stratification. Recently, a sample size of $\geqslant$150 has been defined as a critical threshold for the replication validity of genetic association studies

(Ioannidis *et al*., 2001). So, a power calculation prior to patient recruitment based on the known allele frequency should become an integral part of the planning of any candidate gene association study.

For data analysis, adequate statistical means have to be applied that adjust for all potential co-factors of the disease. In studies with small sample sizes, a Fisher's exact test is appropriate, whereas larger sets of data require the application of a χ^2-test. Most well-analyzed genetic case–control studies performed a multivariate logistic regression analysis which allows for correction of quantitative and qualitative covariates as predictors of the disease outcome. If all these prerequisites are taken into account, large numbers of patients and controls are usually necessary to give a study a sufficient power to detect a significant effect (Cardon and Bell, 2001). This highlights the need for networking in the scientific community and calls for cooperation among different research centers.

PATHOGENESIS OF ALCOHOLIC LIVER DISEASE: POSSIBLE CANDIDATE GENES

Alcohol is hepatotoxic through a variety of mechanisms which lead to acute and chronic tissue injury, and possibly, to cirrhosis. With regard to the liver, alcohol-induced tissue damage is primarily based on the toxicity of its first metabolite acetaldehyde. In addition, the increased formation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-) have been implicated as a cause of liver injury in various forms of chronic liver diseases including ALD (Parola and Robino, 2001). Increased ROS formation is mainly caused by the induction of CYP2E1. The elevated generation of ROS produces cytotoxic oxidative stress and increased lipid peroxidation through the formation of 4-hydroxy 2,3-nonenal (HNE), 4-hydroxy-2,3-alkenals (HAKs) and malondialdehyde (MDA). The capacity of CYP2E1 to oxidize ethanol is increased up to 10-fold in heavy consumers which consecutively increases the pro-oxidative burden (Kessova and Cederbaum, 2003). Defense mechanisms such as antioxidant mitochondrial and cytosolic enzymes can offset some of the toxicity derived from oxidative stress but may become saturated as it persists, or downregulated as liver damage progresses. Furthermore, excessive alcohol consumption can lead to an increased portosystemic uptake of gut-derived endotoxins from gastrointestinal bacteria which contribute to necroinflammation in alcoholic hepatitis by activating Kupffer cells through the CD14/toll-like receptor-4 complex to produce ROS via NADPH oxidase (Parlesak *et al*., 2000; Wheeler *et al*., 2001).

The uniform morphologic response of liver tissue to repeated injury from various sources, including alcohol, is fibrosis which closely resembles the process of scar forming (Bataller and Brenner, 2005). As a response to triggers such as acetaldehyde, ROS, lipid peroxides, and endotoxins, liver macrophages (Kupffer cells) and other inflammatory cells become activated to produce a battery of growth factors and cytokines including the powerful mitogen platelet-derived growth factor (PDGF) and the most important profibrogenic cytokine transforming growth factor β1 (TGFβ1) which stimulate hepatic stellate cells (HSC) and portal fibroblasts (Fig. 1). The transformation of quiescent HSC rich in vitamin A into an activated myofibroblast (MFB)-like phenotype rich in α-smooth muscle actin and devoid of vitamin A is considered the central event in the pathophysiology of liver fibrosis (Bataller and Brenner, 2001). Activated HSCs/MFBs markedly increase the production of extracellular matrix

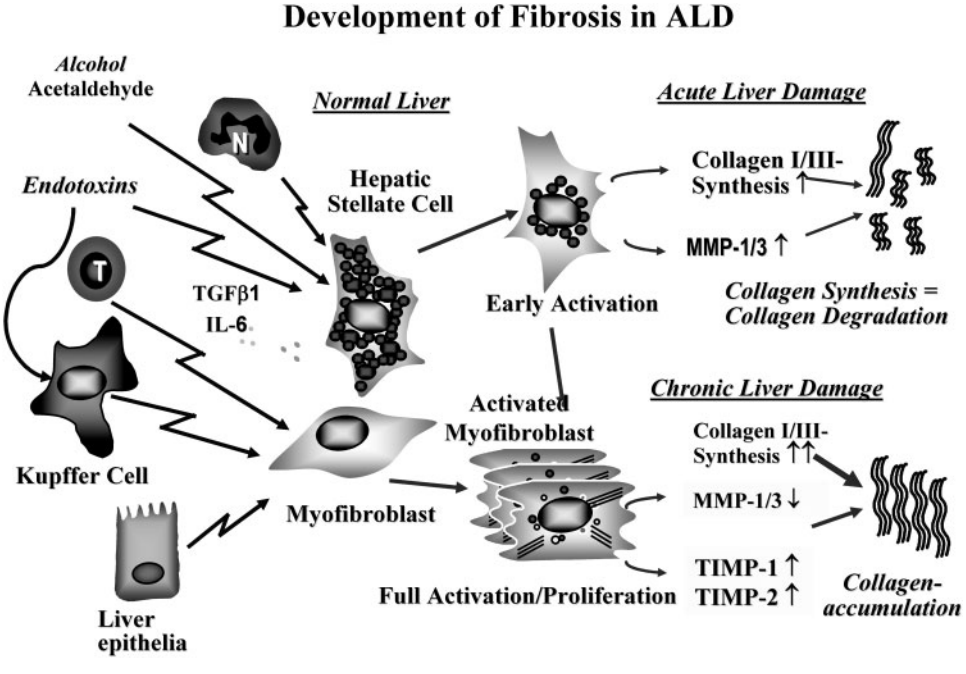

Fig. 1. Alcohol, acetaldehyde, or prooxidants trigger the activation of HSC/myofibroblasts either directly or indirectly through the release of cytokines (IL-1, IL-6, TGF-β1, PDGF). HSC/MFB acquire a profibrogenic phenotype and the ability to migrate, contract, and synthesize ECM. In acute (alcoholic) liver damage, matrix synthesis and degradation is balanced due to concomitant upregulation of matrix-degrading enzymes (MMPs). In chronic liver damage, net matrix accumulation occurs because MMPs are downregulated as a result of upregulated TIMP-1 and TIMP-2 activity.

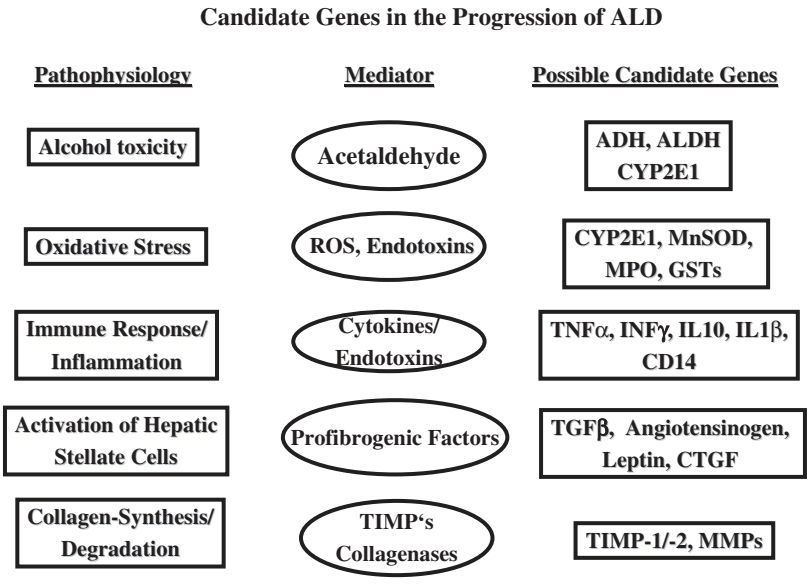

Fig. 2. Genetic susceptibility for the onset or progression of alcoholic liver disease could be related to genetic variation at any step of alcoholic liver damage (e.g. alcohol metabolism, inflammation, and fibrogenesis).

(ECM) molecules which comprise collagens, non-collagenous glycoproteins, proteoglykans, and glycosaminoglykans. However, the accumulation of fibrous material does not simply result from increased production of ECM, but also from its impaired degradation which plays an equally important role (Schuppan *et al*., 2001). In normal liver tissue, a subtle balance is maintained between matrix synthesis and degradation. The latter is exerted by a group of matrixmetalloproteinases (MMPs) released by various liver cells. MMPs process a wide range of ECM substrates (Benyon and Arthur, 2001) and their activity is regulated by a group of specific inhibitors produced by activated HSCs/MFBs termed tissue inhibitors of matrix-metalloproteinases (TIMPs). Upregulation of TIMP-1 and TIMP-2 is responsible for the loss of MMP expression during fibrogenesis in the liver (Herbst *et al*., 1997).

All phases in the development of ALD are regulated by a number genes that either alone or in combination may represent genetic risk constellations that influence the biological reaction towards alcohol. Figure 2 depicts some of the possible genetic candidates that are involved in the pathogenesis of alcohol-related liver injury.

CASE–CONTROL STUDIES ON CANDIDATE GENES IN ALD

In the following, case–control association studies aiming at the identification of genetic risk markers for ALD will be described and weighed regarding their strengths and weaknesses. The description of the studies is divided into sections to pay tribute to the functional role of the tested genes in ALD.

Polymorphisms of genes involved in alcohol metabolism

After absorbtion, alcohol is degraded in the liver and other tissues by alcohol dehydrogenase (ADH) in the cytosol and cytochrome P450 2E1 in microsomes. Acetaldehyde is further converted by aldehyde dehydrogenases (ALDH) to acetate which, after release from the liver, is metabolized by heart and skeletal muscle tissue.

Different classes of ADH isoenzymes are known and a new nomenclature has been introduced recently (Duester *et al*., 1999). As most studies have applied the old ADH classification, it also will be used in this review. All ADHs are dimeric zinc-containing enzymes classified according to their metabolic properties and sequence similarities. Class I ADH comprises isoenzymes with α, β, and γ subunits coded by corresponding gene loci termed ADH1, ADH2, and ADH3 (Bosron *et al*., 1993). Among the human ADH gene loci, two class I ADH genes are polymorphic with three alleles existing for either ADH2 and ADH3 which reveal substantially different enzymatic characteristics. ADH2 alleles are ADH2*1 found in Caucasians and ADH2*2 detectable in Asians which encode the low activity β1 and the high activity β2 subunits, respectively. The resulting dimeric isoenzymes have markedly different k_{cat} values of 9.2 min^{-1} for β1β1 and 400 min^{-1} for β2β2, respectively. The ADH3*1 and ADH3*2 alleles produce the γ1 and γ2 subunits, and the γ1γ1 isoenzyme is twice as active as the γ2γ2 isoenzyme (k_{cat} 87 min^{-1} vs 35 min^{-1}). A recently described ADH3*3 allele has not been enzymatically charaterized (Osier *et al*., 2002).

According to the differences in the capacity to metabolize alcohol to acetaldehyde, it has been speculated that individuals with the more active ADH2*2 and ADH3*1 alleles are at increased risk of developing alcohol-related organ damage due to a higher acetaldehyde exposure (Couzigou *et al*., 1994). The details of population-based case–control studies that have tested a possible association of ADH variants with ALD are summarized in Table 1. Studies were carried out in Asian and Caucasian populations, but due to the fact that the ADH2*2 allele is rare in Caucasians, most data regarding this variant stem from investigations in Asians. However, results are inconsistent. Earlier studies identified the

Table 1. Case–control studies on the association between polymorphisms of alcohol-metabolizing enzymes and ALD

Authors	Tested gene(s)	Sample size	Definition of cases/controls	Functional data	Principle finding
(Couzigou *et al*., 1990)	ADH2, ADH3	46 alcoholic cirrhosis 39 controls	Alcoholic cirrhosis: 100 g alcohol/day for >5 years Signs of portal hypertension	None	No association between either ADH and ALD
(Day *et al*., 1991)	ADH2, ADH3, ALDH2	59 alcoholic cirrhosis 13 alcoholic pancreatitis 79 healthy Subjects	Alcoholic cirrhotics: 80 g alcohol/day for >10 years Liver biopsy in all cirrhotics	None	ADH3*1 more frequent in cirrhotics than in controls (62.7 vs 55.1%, $P < 0.05$)
(Sherman *et al*., 1993)	ADH2	38 ALD 23 healthy controls	Alcoholics: 80 g alcohol/day for >2 years Liver biopsy in all ALD	None	ADH2*B allele associated with ALD
(Chao *et al*., 1994)	ADH2, ADH3, ALDH2	27 alcoholic cirrhosis 23 viral cirrhosis 30 gastroduodenal ulcers	Alcoholic cirrhotics: 80 g alcohol/day for >10 years, esophageal varices Controls: ulcer patients without alcohol consumption	None	ADH2*2, ADH3*1 and ALDH2*2 alleles significantly less frequent in alcoholic cirrhotics than in controls
(Yamauchi *et al*., 1995a)	ADH2, ALDH2, CYP2E1	27 alcoholic fatty liver 7 non-specific changes 46 alcoholic cirrhosis 60 healthy controls	Alcoholics: 120g alcohol/day for >10 years Liver histology in all patients Controls: random	None	ADH2*2/2 genotype associated with alcoholic cirrhosis (OR 4.6; no CI)
(Yamauchi *et al*., 1995b)	ADH2	42 alcoholic cirrhosis 34 noncirrhotic alcoholics	Alcoholics: 120g alcohol/day for >10 years Liver histology in all patients	None	ADH2*2/2 genotype associated with alcoholic cirrhosis
(Tanaka *et al*., 1996)	ADH2, ALDH2	31 ALD 90 alcohol addicts 66 healthy controls	Alcoholics: 80g alcohol/day for >10 years Liver histology in alcoholics Controls: random	None	ADH2*1/1 and ALDH2*1/1 more frequent in alcoholics/ ALD than in controls
(Tanaka *et al*., 1997)	ADH2, ALDH2, CYP2E1	26 ALD 189 controls	Alcoholics: 80g alcohol/day for >10 years Liver histology in alcoholics Controls: random	None	ADH2*1/1, ALDH2*1/1 and CYP2E1 c2 more frequent in ALD than in controls
(Ceni *et al*. 1997)	ADH2, ADH3, ALDH2, CYP2E1	100 alcoholics (26 fatty liver, 29 steatofibrosis, 19 cirrhosis, 26 nonspecific changes)	Alcoholics: 150g alcohol/day or >10 years Liver histology in alcoholics	None	No association with severity between ALD and any of the tested polymorphisms
(Chao *et al*., 1997)	ADH2, ADH3, ALDH2, CYP2E1	75 alcoholic cirrhosis 48 acute pancreatitis 19 heavy drinkers 235 controls	Alcoholics: 60g alcohol/day for >7 years Cirrhosis: CHILD B and C, esophageal varices	None	ADH2*1 and ALDH2*1 more frequent in alcoholic cirrhosis than in controls plus pancreatitis No difference for ADH2 and CYP2E1
(Grove *et al*., 1998)	ADH3, CYP2E1	264 ALD 121 random controls	Alcoholics: 80g alcohol/day for >10 years Liver biopsy 228/264 patients	None	No significant association between ADH3 and CYP2E1, and ALD
(Borras *et al*., 2000)	ADH2, ADH3	180 alcoholic cirrhosis 231 alcoholics 199 viral cirrhosis 224 healthy controls	Alcoholics: 100g (70g) alcohol/day for >10 years Non-alcoholics: <40g (20g)/day	None	Linkage between ADH2*2 and ADH3*1 ADH2*2 allele more frequent in non-alcoholics but overall frequency very low (0–4.8%)
(Monzoni *et al*., 2001)	ADH2, ADH3, CYP2E1, TNFα	158 alcoholics	Alcoholics: 120g (60g) alcohol/day (questionnaire)	None	ADH3*2 and CYP2E1 c2 allele associated with severity of ALD

Table 1. *Continued*

Authors	Tested gene(s)	Sample size	Definition of cases/controls	Functional data	Principle finding
(Lee *et al*., 2001)	ADH2, ADH3, ALDH2, CYP2E1	56 alcoholic cirrhosis 52 alcoholics without ALD 64 non-drinkers	Alcoholics: 80g alcohol/day for >10 years Cirrhosis: ascites, esophageal varices, encephalopathy	None	No association with severity between alcoholic cirrhosis and any of the tested Genes
(Frenzer *et al*., 2002)	ADH2, ADH3, ALDH2, CYP2E1, ApoE GSTM1/T1,	57 alcoholic cirrhosis 71 alcoholic pancreatitis 57 alcoholics 200 blood donors	Alcoholic cirrhosis: biopsy-proven or clinical evidence	None	ADH3*2/2 genotype more frequent in cirrhotics than in blood donors All cirrhotics had ADH2*1/1
(Vidal *et al*., 2004)	ADH2, ADH3, CYP2E1	99 alcoholic cirrhosis 118 ALD without cirrhosis 47 alcoholics 255 non-alcoholics (healthy, liver disease, cirrhosis)	Alcoholics: 100 g alcohol/day for >10 years Liver histology in alcoholics Non-alcoholics: <20 g/day	None	No association with severity between ALD and any of the tested Polymorphisms
(Tsutsumi *et al*., 1994)	CYP2E1 (RsaI + PstI)	50 ALD 10 alcoholics without ALD 34 Non-ALD 88 Non-liver disease 66 Healthy controls	Alcoholics: 80g alcohol/day for >5 years Liver biopsy in all ALD No characterization of controls	Acetaminophen elimination test showed higher activity in c2 homozygotes	Alcoholic cirrhotics had higher rate of mutant c2 allele and c2 homozygosity
(Maezawa *et al*., 1994)	CYP2E1 (RsaI + PstI)	20 alcoholic steatosis 62 alcoholic fibrosis/ cirrhosis	Alcoholics: 120 g alcohol/day for >10 years Liver biopsy in all ALD	None	Genotype CYP2E1 c1/c1 more frequent in advanced ALD
(Chao *et al*., 1995)	CYP2E1 (RsaI + PstI)	54 alcoholic cirrhosis 23 alcoholic pancreatitis 33 non-liver disease 31 viral cirrhosis 100 healthy controls	Alcoholics: 80g alcohol/day for >8 years All cirrhotics had decompensated liver disease	None	No association of CYP2E1 allels with alcoholic cirrhosis marked ethnic differences between Asians and Caucasians/ African Americans
(Pirmohamed *et al*., 1995)	CYP2E1 (RsaI + PstI)	95 ALD 58 alcoholics without ALD 47 Non-ALD 100 healthy subjects	Alcoholics: 190g alcohol/day (median) > 10 years 33% of ALD with biopsy	None	c2 allele more frequent in ALD (OR 4.5; CI 1.9-10.9)
(Carr *et al*., 1995)	CYP2E1 (RsaI + PstI)	53 ALD (hepatitis and cirrhosis) 21 alcoholics without ALD 18 recovering alcoholics 32 hospital staff member	ALD: average intake 257g alcohol for 27 years Alcoholism: DSM II criteria	None	No association of CYP2E1 alleles with alcoholic cirrhosis
(Ball *et al*., 1995)	CYP2E1 (RsaI + PstI)	37 alcoholic cirrhosis 24 Korsakoff's syndrome 39 alcohol dependence 108 matched controls	Alcoholics: 80g alcohol/day > 3 years Liver biopsy in all ALD	None	No association of CYP2E1 alleles with alcoholic cirrhosis
(Carr *et al*., 1996)	CYP2E1 (RsaI + PstI)	Han Chinese: 30 alcoholic cirrhotics 46 alcoholics without ALD 100 random controls Atayal Taiwanese: 34 alcoholics 35 normal controls	Alcoholics: 80g alcohol/day > 10 years Alcoholism: DSM II criteria	None	No association of CYP2E1 alleles with alcoholic cirrhosis and alcoholism

Table 1. *Continued*

Authors	Tested gene(s)	Sample size	Definition of cases/controls	Functional data	Principle finding
(Lucas *et al*., 1996)	CYP2E1 (RsaI, DraI), CYP1A1 (MspI)	202 alcoholics without ALD 110 alcoholic cirrhosis 62 esophageal cancer 96 UADT cancer 41 alcoholic disorders	Alcoholic cirrhosis confirmed through biopsy Assessment of alcohol consumption not described	None	No association of CYP2E1 alleles with alcoholic cirrhosis DraI SNP more frequent among alcoholics
(Agundez *et al*., 1996)	CYP2E1 (RsaI)	58 alcoholic cirrhosis 137 normal controls	Alcoholics: 100g alcohol/day for >10 years	None	No association of CYP2E1 RsaI SNP with alcoholic cirrhosis
(Savolainen *et al*., 1997)	CYP2E1 (RsaI, PstI, DraI, MspI)	Male autopsy cases 243 alcoholics 43 moderate drinkers 33 controls	Alcoholics: >80 g alcohol/day Moderate drinkers: 10–80 g Controls: 0–10 g	None	No association of CYP2E1 alleles with ALD RsaI + PstI very infrequent
(Parsian *et al*., 1998)	CYP2E1 (RsaI + PstI)	174 alcoholics (43 cirrhosis) 89 controls	Alcoholics: Cloninger criteria Liver histology in all cirrhotics	None	No association of CYP2E1 alleles with alcoholic cirrhosis or alcoholism
(Rodrigo *et al*., 1999)	CYP2E1 (PstI), ADH2, NAT2, GSTM1	120 alcoholic cirrhosis 30 alcoholics without ALD 200 healthy controls	Alcoholics: 200g alcohol/day > 20 years	None	NAT2*5 allele (slow acetylator status) decreased in alcoholic cirrhotics Other SNPs not related to cirrhosis
(Itoga *et al*., 1999)	9 exons and exon- intron junctions of CYP2E1	44 ALD 96 alcoholics without ALD 115 healthy controls	ALD: liver biopsy in all ALD patients Alcoholism: DSM III-R Controls: non-drinking, light consumption (22–44g/day > 5 years), moderate consumption (>66g/day > 5 years)	None	No association between any nucleotide replacement and ALD
(Wong *et al*., 2000)	CYP2E1 (RsaI, PstI, TaqI)	61 ALD 46 HCC 375 healthy controls	Alcoholics: 60g alcohol/day for >10 years Liver biopsy in all ALD subjects	None	No association between CYP2E1 RsaI and PstI SNPs and alcoholic cirrhosis TaqI SNP protective towards alcoholc cirrhosis
(Pastorelli *et al*., 2001)	CYP2E1 (RsaI + PstI), DRD2 and SLC6A4	60 alcoholics (18 cirrhosis) 64 healthy control	Alcohol quantification via interview	None	No association with severity between ALD and any of the tested polymorphisms
(Plee-Gautier *et al*., 2001)	CYP2E1 (1C + 1D alleles)	98 alcoholic cirrhosis 148 alcoholics without ALD 103 random controls	Alcoholics: 80g alcohol/day for more than 'several' years	Chlorzoxazone test	No association between CYP2E1 1C and 1D alleles and alcoholic cirrhosis

ADH2*2 allele and genotype ADH2*2/2 as a risk factor for ALD (Sherman *et al*., 1993; Chao *et al*., 1994; Yamauchi *et al*., 1995a, 1995b), whereas subsequent investigations rather found the allele ADH2*1 and genotype ADH2*1/1 to be associated with ALD (Tanaka *et al*., 1996, Tanaka *et al*., 1997; Chao *et al*., 1997; Frenzer *et al*., 2002). Among the former it is noteworthy to mention, that Yamauchi *et al*. (Yamauchi *et al*., 1995a, 1995b) were seemingly able to publish the same data twice. While several investigators reported associations between the ADH3*2 allele or the ADH3*2/2 genotype, respectively, and ALD (Monzoni *et al*., 2001; Frenzer *et al*., 2002), others suggested a role of the high turnover allele ADH3*1 to confer risk to the development of ALD (Day *et al*., 1991). However, a number of studies found no association at all (Table 1). The true role of ADH genotypes in the risk of ALD is difficult to define since many of the published studies reveal important limitations as regards the interpretability of the data. For example, most studies reveal a very low sample size and, therefore, a lack of statistical power likely hampers the significance of the key finding. 'Underpowered' investigations are highly susceptible towards chance observations which may explain the discrepancies between many of the studies. Also, relatively simple tests were used for statistical analyses, such as mere counting of alleles and genotype frequencies, Student's *t*-tests and Fisher's exact tests which are inappropriate for determining the influence of certain factors in complex settings. Only one study has applied a multiple logistic

regression analysis that takes numerous modifiers of disease into account (Yamauchi *et al*., 1995b). Morerover, no study presented *in vitro* or *in vivo* data supporting the functional significance of the association and the hypothesis, such as the testing for differences in acetaldehyde levels in individuals with different genotypes. Overall, the available data does not provide clear evidence that demonstrates a contribution of ADH genotypes to the development of ALD.

A similar situation prevails with regard to genetic variants of CYP2E1. In contrast to ADH and ALDH, CYP2E1 is an inducible enzyme and its activity can increase up to 20-fold following continuous alcohol consumption. Therefore, functional polymorphisms are just one possible factor in the individual variation of CYP2E1 activity. There are several polymorphic loci within the human CYP2E1 gene of which four are identifiable via RFLP (Hayashi *et al*., 1991). Two mutations were found to be in linkage disequilibrium, giving rise to the c1 and c2 allele (Watanabe *et al*., 1990). The CYP2E1 c2 (mutated) allele is associated with an up to 10-fold higher gene transcription, protein level, and enzyme activity than the c1 allele and could result in a higher exposure of the liver towards acetaldehyde and ROS (Watanabe *et al*., 1994). Therefore, CYP2E1 is an interesting candidate gene in case–control association studies and published reports are listed in Table 1. However, many of the limitations outlined for ADH and ALDH genotype association studies also hold true for studies investigating CYP2E1 SNPs including lack of statistical power, insufficient characterization of cases and controls, inappropriate statistical analysis, and lack of functional data. Altogether, a significant contribution of CYP2E1 variants to the emergence of ALD is unlikely.

While both ADH3 and CYP2E1 genotypes do not seem to be a strong risk factor of progression of ALD, their significant contribution to the development of HCC was suggested by two reports which showed that individuals homozygous for the alleles CYP2E1 c1 (Yu *et al*., 1995) and ADH3*1/ADH1C*1 (Homann *et al*., 2006) are at an increased risk to develop hepatoma. In the latter study, 818 alcoholics either with or without alcohol-related disorders including liver cirrhosis, chronic pancreatitis, alcohol-related carcinoma of the esophagus, head and neck, and liver ($n = 86$) were genotyped for ADH3/ADH1C variants. The odds ratio for genotype ADH3*1/1 regarding the development of HCC was 3.56 (CI 1.33–9.53) and multivariate analysis identified the ADH3*1 allele and its homozygosity as independent risk factors for HCC in heavy drinkers with preexisting cirrhosis.

Genetic polymorphisms of antioxidant enzymes

Numerous lines of evidence point to an important role of oxidative stress in the pathogenesis of ALD. During alcohol metabolism, ROS are generated as a result of the production of NADH from the conversion of ethanol to acetaldehyde by ADHs, and of NADPH from the metabolism of ethanol by CYP2E1. ROS are highly reactive and can damage lipids, proteins, and DNA (Arteel, 2003). Several enzymes exist which counteract oxidative stress generated in ALD including glutathione-*S*-transferases (GSTs) and superoxide dismutase. GSTs are expressed in the liver and other organs and comprise several gene subfamilies encoding sulphur-containing enzymes which inactivate ROS and many toxic and carcinogenic xenobiotics through conjugation with glutathione (GSH) (Armstrong, 1997). GST isoenzymes reveal profound differences in their structure and substrate specificity and >30 polymorphic variants have been identified (Hayes *et al*., 2005). Among these, polymorphisms of the α (GSTA), μ (GSTM), τ (GSTT), and π (GSTP) classes have been studied in patients with ALD and the major characteristics of the published reports are outlined in Table 2.

Most case–control studies investigated the GSTM1 and GSTT1 genes due to the existence of 'null' allelic variants resulting from a partial deletion in either gene locus with absence of enzyme activity. Consequently, a loss or deficiency in GSTM1 and GSTT1 enzyme activity could potentially increase the levels of toxic intermediates generated along with chronic alcohol consumption. However, only few studies have found an association between 'null' genotypes of GSTM1 and GSTT1, and ALD (Groppi *et al*., 1991; Ladero *et al*., 2005), while several reports showed no relation (Savolainen *et al*., 1996; Rodrigo *et al*., 1999; Frenzer *et al*., 2002; Burim *et al*., 2004; Hayes *et al*., 2005). However, in the largest study, a significantly increased frequency of GSTM1 'null' genotype in heavy drinkers with advanced ALD has been demonstrated (Savolainen *et al*., 1996). Moreover, a recent study from Spain showed an increased risk for ALD in individuals with combined carriage of GSTM1 and GSTT1 'null' genotype (Ladero *et al*., 2005). Although attractive, genetic polymorphisms of GST isoenzymes cannot be considered as firmly established since findings are still conflicting or have been generated in studies with an insufficient design. Our own findings in a larger case–control series indicate no risk of GSTP1 polymorphisms (Eurich, D., Friess, H., Hellerbrand, C., Homann, N., Kolb, A., Österreicher, C. H., Patsenker, E. *et al*., unpublished data). However, we have recently found genotype GSTP1 Val/Val to be associated with the development of cirrhosis in patients with hereditary hemochromatosis (Stickel *et al*., 2005). As in ALD, oxidative stress plays an important role in the pathogenesis of hemochromatosis.

Hepatocyte mitochondria are prime targets of oxidative stress generated in chronic alcoholism and alterations of mitochondrial function and structure, such as the breakdown of mitochondrial membrane potential, have been recognized as key events in the onset of alcohol-driven apoptosis (Adachi and Ishii, 2002). The ability to resist oxidative pressure largely depends on the mitochondrial GSH content and antioxidant mitochondrial enzymes. Mitochondria-derived ROS are detoxified to hydrogen peroxide and water by the successive action of manganese superoxide dismutase (MnSOD) and GSH peroxidase, respectively (Wallace, 1999). MnSOD is synthesized with a cleavable N-terminal mitochondrial target sequence that enables its transport into mitochondria (Shimoda-Matsubayashi *et al*., 1996). A SNP within codon 16 of the precursor protein leads to either alanine (Ala) or valine (Val) at amino acid position –9 of the target sequence resulting in enhanced translocation into mitochondria and higher concentration of active MnSOD in case of the Ala-sequence (Sutton *et al*., 2003). Table 2 summerizes case–control studies that investigated MnSOD variants.

Degoul *et al*. (2001) have genotyped 71 patients with ALD stratified according to the degree of liver damage and have found that the Ala/Ala genotype occurs more frequently in

Table 2. Case–control studies on the association between polymorphisms of antioxidant enzymes and ALD

Authors	Tested gene(s)	Sample size	Definition of cases/controls	Functional data	Principle finding
(Groppi *et al*., 1991)	GSTM1	45 alcoholic cirrhosis 45 healthy controls	Cirrhotics: >100 g alcohol/day for >5 years Presence of ascites, varices	None	No relationship between alcoholic cirrhosis and GSTM1 genotypes
(Savolainen *et al*., 1996)	GSTM1	313 alcoholics 43 moderate drinkers 33 control subjects	Alcoholics: >80 g alcohol/day Moderate drinkers: 10–80 g Controls: 0–10 g	None	GSTM1 'null' genotype more frequent among ALD with advanced fibrosis (OR 2.3; CI 1.11-4.76)
(Rodrigo *et al*., 1999)	GSTM1, CYP2E1 (PstI), ADH2, NAT2,	120 alcoholic cirrhosis 30 alcoholics without ALD 200 healthy controls	Alcoholics: 200 g alcohol/day for >20 years	None	GSTM1 polymorphism not related to cirrhosis
(Frenzer *et al*., 2002)	GSTM1/T1, ADH2, ADH3, ALDH2, CYP2E1, ApoE	57 alcoholic cirrhosis 71 alcoholic pancreatitis 57 alcoholics 200 blood donors	Alcoholic cirrhosis: biopsy-proven or clinical evidence	None	No association between alcohol-related disorders and GST genotypes
(Burim *et al*., 2004)	GSTM1, T1, P1, CYP2E1, CYP1A1	65 alcoholic cirrhotics 14 alcoholic pancreatitis 41 alcoholics 221 non-alcoholic controls	Alcoholics: >40g alcohol/day obtained from case records Mixed ethnicity	None	GSTs not associated with any of the endpoints CYP1A1 associated with cirrhosis (OR 5.33; CI 1.23–23.14)
(Brind *et al*., 2004)	GSTM1, M3, P1, T1, A1	Unclear number of healthy controls, alcoholics without ALD, and ALD patients	Alcoholics: different definitions in three centers	None	No association between ALD and any of the tested SNPs
(Ladero *et al*., 2005)	GSTM1, T1	153 ALD 241 random controls	Alcoholics: 150 g alcohol/day (median) for >10 years Cirrhosis: clinical and imaging evidence of portal hypertension	None	GSTT1 'null' genotype associated with ALD (OR 1.67; CI 1.03–2.71) Carriers of GSTT1 and M1 'null' genotypes are at increased risk for ALD (OR 4.3; CI 1.89–9.97)
(Degoul *et al*., 2001)	MnSOD	71 ALD 79 blood donors	Average alcohol intake 151 g (±98)/day for 18 ± 6 years Liver biopsy in all ALD patients	None	Rate of MnSOD Ala homozygos ity increased significantly with severity of ALD (RR of cirrhosis for Ala/Ala 9.6; CI 2.6–35.4)
(Stewart *et al*., 2002)	MnSOD	281 advanced ALD 218 alcoholics without ALD 244 healthy controls	Alcoholics: 80g alcohol/day for >10 years ALD: clinical and biochemical evidence, imaging Controls: alcohol <21 U (men), <15 U (women)	Circulating antibodies raised against markers of oxidative stress	No difference in MnSOD geno type and allele frequencies between groups. Serum markers of oxidative stress similar in different genotypes
(Brind *et al*., 2003)	MnSOD	357 ALD 93 alcoholics without ALD 474 non-drinking controls	Alcoholics: different definitions in three centers	None	No difference in MnSOD geno type and allele frequencies between groups
(Nahon *et al*., 2005)	MnSOD	Longitudinal study in 264 alcohol cirrhotics	Alcoholic cirrhosis: liver biopsy, for >80 g alcohol/day, alphafetoprotein <50 ng/ml	None	RR for HCC with 1 Ala allele 4.59 (CI 1.61–13.06) RR for death due to cirrhosis with 1 Ala allele 2.49 (CI 1.36–4.57)

patients with severe ALD. They assessed an OR of 9.6 (CI 2.6–35.4) for genotype MnSOD Ala/Ala for the presence of cirrhosis. However, the study included only 13 cirrhotic patients. Although the authors did not provide a power calculation, there is an obvious lack of statistical power due to a small sample size. The same group later presented data from a longitudinal study suggesting that the presence of at least one Ala MnSOD allele increases the risk for developing cirrhosis in French subjects and, furthermore, with the development of HCC and death due to cirrhosis (Nahon *et al*., 2005). However, case–control studies from other researchers with larger numbers of patients and controls could not confirm these findings (Stewart *et al*., 2002; Brind *et al*., 2003).

Polymorphic genes coding for cytokines involved in ALD

Compelling evidence points to an important role of the immune system in mediating alcoholic liver injury. The innate immune response acts as the first line of nonspecific defense against exogenous pathogens such as ROS, lipid peroxides, and endotoxins/lipopolysaccharides derived from the outer cell membrane wall of gram negative bacteria in the intestine which activate immune effector cells. Particularly, Kupffer cells play an important role and experimental inhibition of Kupffer cells prevents an array of hepatic reactions in response to ethanol including the elevation of serum transaminases, steatosis, inflammation, and necrosis (Hines and Wheeler, 2004). Numerous experimental studies found that Kupffer cells are stimulated by various triggers to produce a number of cytokines including tumor necrosis factor α (TNFα), a proinflammatory cytokine known to potentially cause hepatocyte death. Apart from TNFα, several other cytokines including interleukins, interferons, chemokines, and certain growth factors regulate hepatic inflammation, apoptotic and necrotic cell death, cholestasis, and fibrosis (Tilg and Diehl, 2000). For several genes that code for proteins involved in these processes, polymorphisms with functional implications have been detected that render these variants interesting candidates for case–control association studies.

Excess TNF-α production is a typical feature of ALD. The biological response is dependent on the level of TNF-α the liver is exposed to and may result in increased hepatocyte proliferation, activation of cell survival factors such as upregulation of MnSOD and bcl-x_L, or cell death through initiation of apoptosis or necrosis. Therefore, polymorphic variation of the TNF-α gene leading to variable TNF-α levels may influence TNF-α-dependent inflammation following alcohol intake. In fact, two SNPs at position –308 (G→A) and –238 (G→A), respectively, of the TNF-α gene are associated with increased TNF-α expression thereby possibly influencing the progression of ALD (Wilson *et al*., 1993; D'Alfonso and Richiardi, 1994). Grove and co-workers (Grove *et al*., 1997) were the first to study these two TNF-α polymorphism in a cohort of patients with ALD. While the distribution of the polymorphism at position –308 was not different between the cases and controls, an excess of the rare TNFA-A allele at position –238 was found in patients with ALD. The OR for this variant vs non-diseased patients was 3.5 (0.4–28) with regard to cirrhosis and 4 (1.2–14) for alcoholic steatohepatitis. All patients had biopsy-proven ALD and the distribution of genotypes was in Hardy–Weinberg equilibrium in all groups. However, the authors did not provide evidence that the tested polymorphisms resulted in differences in TNF-α serum levels, neither did they show SNP-dependent variation in hepatic TNF-α expression. Two subsequent cohort studies also tested TNF-α variants but found no association (Bathgate *et al*., 2000; Ladero *et al*., 2002). However, one study testing the TNF-α –308 SNP investigated only 25 patients with ALD and, therefore, could have missed a relationship due to low sample size (Bathgate *et al*., 2000). The other study included a larger number of ALD patients but was carried out in Spain, so ethnic differences may have contributed to the discrepancy (Ladero *et al*., 2002). The latter two studies also included several polymorphisms in the interleukin-10 (IL-10) gene which alter gene transcription and IL-10 serum concentrations. IL-10 has emerged as an important inhibitor of inflammatory responses such as the downregulation of proinflammatory cytokines including IL-1, TNF-α, IL-6, IL-8, and IL-12. Moreover, IL-10 was shown to upregulate the expression of the IL-1R antagonist, to inhibit collagen gene transcription, and to increase collagenase expression in HSC (Wang *et al*., 1998). However, no association was found in contrast to a previous report in 287 patients with biopsy-proven ALD in which the possession of the A allele in the IL-10 promoter was associated with an increased risk of advanced ALD (Grove *et al*., 2000).

Two reports are available on the association of a polymorphism in the interleukin-1 receptor antagonist (IL-1Ra) gene with ALD (Takamatsu *et al*., 1998; Pastor *et al*., 2000). IL-1Ra is a potent antiinflammatory cytokine that can inhibit immune-mediated inflammatory reactions. IL-1Ra relates to IL1 which binds to its corresponding receptor IL-1 (IL-1R). A variable nucleotide tandem repeat polymorphism resulting in different allele sizes due to variable numbers of repeats (A1: 4 repeats; A2: 2 repeats; A3: 5 repeats; A4: 3 repeats) was shown to alter IL-1Ra expression in monocytes *in vitro* (Danis *et al*., 1995). One study included Spanish alcoholics with alcohol addiction, significant alcohol abuse, and alcoholic cirrhosis, respectively (Pastor *et al*., 2000). While the presence of the A1 allele increased the risk for alcoholism, no relationship with ALD was detected. In the Japanese study, genotype and allele distributions differed from that in Caucasians and heterozygosity for the A1 allele was more frequent in alcoholics with fibrosis than in those without (Takamatsu *et al*., 1998). However, this difference did not reach statistical significance. Owing to the low number of cases, the findings of the latter study are highly suspicious of being chance observations which particularly occur in genotypes with low frequencies (Day, 2003).

In addition, one study each was performed to investigate a possible relationship between poylmorphisms of interleukin-1β (IL-1β), a promoter polymorphism of the CD14 endotoxin receptor, and the cytotoxic T-lymphocyte antigen-4 gene (CTLA-4), and ALD. The details of studies on polymorphic variation of cytokine genes in the development of alcoholic hepatitis and cirrhosis are outlined in Table 3. All three reports found associations between certain genotypes and the development of alcoholic cirrhosis; however, no other study has so far repeated, let alone confirmed these findings.

Table 3. Case–control studies on the association between polymorphisms of cytokines and immune factors, and ALD

Authors	Tested gene(s)	Sample size	Definition of cases/controls	Functional data	Principle finding
(Grove *et al*., 1997)	TNFα	150 ALD (cirrhosis, ASH) 145 controls	Alcoholics: 80 g alcohol/day >10 years Liver biopsy in all ALD	None	–238 TNF-α G/A genotype associated with cirrhosis (OR 3.5; CI 0.4–28) and ASH (OR 4; CI 1.2–14)
(Takamatsu *et al*., 1998)	IL-1R antagonist	46 alcoholic cirrhosis 21 alcoholic fibrosis 35 alcoholics without ALD 46 healthy subjects	Alcoholics: 120g alcohol/day > 10 years Liver biopsy in all ALD	None	IL-1Ra A1 heterozygotes more frequent in fibrosis/cirrhosis Cumulative alcohol intake lower in A1 carriers with alcoholic fibrosis
(Grove *et al*., 2000)	IL-10	287 advanced ALD 107 NALD 227 healthy subjects	Alcoholics: 80 g alcohol/day >10 years ALD: clinical and biochemical evidence, imaging, biopsy	None	Carriage of A allele (–627) of IL-10 promoter associated with advanced ALD (OR 2.04; CI 1.42–2.92)
(Bathgate *et al*., 2000)	TNFα, IL-10, TGFβ1	25 ALD 113 non-alcoholic end-stage liver disease (viral, cholestatic, autoimmune, acute liver failure)	All patients transplant recipients Alcohol consumption not detailed	None	None of the tested genotypes associated with ALD
(Ladero *et al*., 2002)	TNFα, IL-10	147 advanced ALD 355 control subjects	Alcoholics: 150 g alcohol/day (mean) >10 years ALD: clinical evidence, imaging results, endoscopy, biopsy (n = 12)	None	Single SNPs not related to ALD Excess of G11-GCC haplotype in ALD(OR 2.08; CI 1.31-3.31)
(Takamatsu *et al*., 2000)	IL-1β	142 ALD (steatofibrosis, cirrhosis, alcoholic hepatitis)	Alcoholics: 120 g alcohol/day for >10 years Liver biopsy in all ALD	None	Carriers of –511 IL-1β allele 2 more frequent among alcoholic cirrhotics (OR 2.3; CI 1.1–4.8) Haplotype IL-1β –511 allele 2/+3953 allele 1 associated with alcoholic cirrhosis
(Järvelainen *et al*., 2001)	CD14-Endotoxin receptor	48 alcoholic cirrhosis 39 alcoholic hepatitis 59 alcoholic fibrosis 108 steatosis 178 normal liver	Alcoholics: 40g alcohol/day for 24.9 (±10.8) years ALD: autopsy	None	T allele of CD14-Endotoxin receptor associated with alcoholic hepatitis (OR 2.48; CI 1.17–5.24) and alcoholic cirrhosis (OR 3.45; CI 1.49–7.99)
(Valenti *et al*., 2004)	CTLA-4	183 ALD (cirrhosis, fibrosis, steatosis) 115 chronic hepatitis C 102 Non-alcoholic fatty liver disease	Alcoholics: 60 g (men)/ 40 g (women) alcohol/day for >5 years Alcoholic cirrhosis assessed with biopsy in 128/183	None	Genotype CTLA-4 G/G associated with ALD (OR 3.5; CI 1.1–11)
(Pastor *et al*., 2000)	IL-1R antagonist	30 alcoholic cirrhosis 30 alcohol abusers 30 alcohol dependent men	Alcoholics: 100g alcohol/day for >5 years Alcoholic cirrhosis: biopsy (n = 24), biochemical evidence (n = 6)	None	Presence of the A1 IL-1R antagonist allele associated with alcoholism but not with alcoholic cirrhosis
(Oliver *et al*., 2005)	TGFβ1	165 advanced ALD 185 controls	Alcoholics: 167 g alcohol/day (mean) >10 years ALD: clinical evidence, imaging results, endoscopy, biopsy (n = 16)	None	No association between tested SNP and ALD

Genetic variants of genes relevant for fibrogenesis and fibrolysis

Genes that govern the production and degradation of fibrous tissue are interesting with regard to many etiologies of chronic liver disease since the formation of fibrosis in different disease entities share many similarities (Friedman, 2000). These genes could represent genetic markers of progression rather than of the susceptibility towards a certain disease trigger since fibrosis develops with a long latency that is unlikely to influence behavioral aspects of alcoholism. Consequently, genes that were identified to confer risk to advanced liver damage in one form of chronic liver disease is likely to be involved in another etiology as well. Accordingly, several candidate genes that are involved in connective tissue turnover have

been tested in other diseases, such as TGFβ1 in chronic hepatitis C (Powell *et al*., 2000) and hemochromatosis (Österreicher *et al*., 2005), MMPs in primary sclerosing cholangitis (Satsangi *et al*., 2001), and TIMPs in the development of asthma and arterial aneurysms (Krex *et al*., 2003; Lose *et al*., 2005).

With regard to ALD, only data from studies on the role of TGF-β1 polymorphisms are available so far (Table 3). Two studies have analyzed several TGF-β1 polymorphisms which lead to elevated TGF-β1 expression in the setting of ALD, neither found an association with alcoholic cirrhosis (Bathgate *et al*., 2000; Oliver *et al*., 2005). Our own data on a polymorphism at codon 25 of the signal sequence (Arg→Pro) within the TGFβ1 gene resulting in higher TGFβ1 levels showed no difference in a cohort of 153 alcoholic cirrhosis compared with a cohort of 118 matched heavy drinkers without liver damage (Eurich, D., Friess, H., Hellerbrand, C., Homann, N., Kolb, A., Österreicher, C. H., Patsenker, E. *et al*., unpublished data).

With regard to the progression of fibrosis, MMPs qualify as ideal candidates since their function is closely linked to the accumulation of ECM. Among several MMPs that are expressed in the liver, MMP-3 (stromelysin) is crucial because of its capacity to degrade a broad spectrum of ECM molecules and to activate other MMPs. The presence of 5 adenosines (5A) instead of 6 adenosines (6A) at bp –1171 results in increased MMP-3 activity (Ye *et al*., 1996). As mentioned above, a recent study found an association between genotype MMP-3 5A/5A and the progression of liver damage in patients with primary sclerosing cholangitis (Satsangi *et al*., 2001), but its role in ALD is unknown. In a recent study from our own group, MMP-3 5A/5A genotype was found more frequently in alcoholic cirrhotics than in those without (30.1 vs 15.3%, $P = 0.017$), and multivariate analysis identified age and genotype MMP-3 5A/5A as independent risk factors for alcoholic cirrhosis (Stickel *et al*., manuscript in revision). The adjusted odds ratio of genotype MMP-3 5A/5A for the development of cirrhosis was 1.52 (95% CI 1.108–2.086, $P = 0.010$). Notably, RT–PCR revealed significantly higher MMP-3 transcription in individuals with MMP-3 5A/5A genotype compared with the MMP-3 5A/6A (6A/6A) variant. However, whether the MMP-3 5A/5A genotype represents the first fibrosis-associated genetic risk factor for the progression of ALD remains to be confirmed in an independent cohort of alcoholic cirrhotics.

DESIGN OF GENETIC ASSOCIATION STUDIES

Many published genotype–phenotype association studies were carried out based on exciting hypotheses but collected questionable genetic data since important requirements to design and statistical interpretation were not met. Consequently, not only results were published that could not be reproduced due to lack of a true association with the studied disease, but also some investigations may have even missed an association because of an insufficient approach. Frequently encountered problems of previous case–control association studies in many diseases include the lack of statistical power due to small sample size (Ioannidis *et al*., 2003), population stratification (Cardon and Bell, 2001), ethnic heterogeneity (Deng, 2001), deviation form Hardy–Weinberg equilibrium due to errors in patient and control selection or genotyping, and lack of control for confounding factors (Colhoun *et al*., 2003). Consequently, replication validity of genetic association studies has been unsatisfactory because results of the first study rarely correlated with subsequent findings. This discrepancy is more likely to occur as more studies are performed on a potential association and when the sample size of the first study has been low (Ioannidis *et al*., 2001). Notably, 'index' studies on a genotype–phenotype association are often published in prestigious journals that yield high impact factors, whereas subsequent research with often better design face problems to get accepted at all, or appear in journals with lower average impact factors. The same accounts for the calculated odds ratios for a given association: while the first study indicated impressive estimates of predisposition against or protection from a certain disease, subsequent data tended to show much less pronounced relationships or even the opposite (Ioannidis *et al*., 2001).

In order to avoid these mistakes and to prevent much energy and financial resources from being wasted on time-consuming and, lastly, unreproducible efforts, some recommendations are proposed for the design of genetic case–control association studies (Table 4).

CONCLUSION

Agreement exists that the manifestation of ALD is partly determined by genetic factors and substantial efforts have

Table 4. Recommendations for design and data analysis of genetic case–control associaiton studies

	Recommendation
Candidate gene	Define a convincing rationale for the selection of the studied gene(s)
	Inlude several genes with related roles in disease pathogenesis or common haplotypes with which the gene of interest cosegregates
	Validate reliability of genotyping procedure by repeated blinded testing
Genetic variant	Ensure functional implication of the genetic variant and make sure it leads to a plausible hypothesis
	Refer to published functional evidence of the studied polymorphisms, or, if not available, include own *in vitro* or *in vivo* data to support association with functional differences
Selection of cases and controls	Define cases and controls according to standardized criteria that allow correction for covariates of the disease (exposure to trigger, comorbidities).
	Consider important demographic factors (ethnicity, age, gender)
	Recruit patients and controls prospectively
	Attempt to confirm key finding in an independent cohort of cases and controls recruited along the same strategy
Data analysis	Perform power calculation prior to recruitment to avoid 'underpowered' studies
	Evaluate data by applying a multivariate logistic regression analysis that corrects for potential covariates and minimizes confounding effects
	Check whether the observed association is in agreement with the function of the genetic variant and whether there is a dose-response effect

been made to identify such genetic modifiers, mostly by means of genetic case–control association studies. SNPs of genes that code for proteins that play a role in the pathogenesis of ALD were tested. So far, results are conflicting and initial euphoria over seemingly identified genetic markers has faded since many results could not be reproduced. Therefore, future studies have to adopt certain criteria that assure statistical power via large-scale multicenter cooperations, functional relevance of the tested genes/haplotypes, and consider functional proteomics and genomics. Bearing this in mind, research efforts will contribute to better disease management and patients' benefit.

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